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| Applicant(s): | Pastorello et al. | Confirmation No.: | 5027 |
| Serial No.: | 10/580,659 | Art Unit: | 1651 |
| Filed: | May 26, 2006 | Examiner: | Kosar, Aaron J. |
| Title: | "Composite structures containing hyaluronic acid the derivatives thereof as new bone substitutes and grafts" | | |

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

I, Anna Zanellato, being duly sworn depose and say that:

1. I am an Italian citizen residing at: Bovolenta (PD)

2. I am familiar with the English language.

3. I graduated in: BIOLOGY at the University of Padua in the academic year: 1987

4. I am author of 19 Scientific publications and co-inventor of 4 patents.

5. Previous job experiences: From 1987 to 1990 I had worked at the University Department of General Pathology as a researcher, where I had been involved in a study pertaining to smooth muscles cells cultures and in particular to the mechanism of atherosclerosis.

6. Actual job: Since 1990 I have been working at FIDIA FARMACEUTICI S.p.A. in the field of research, involving:

- the analysis of action mechanism of trophic factors,
- studies, utilizing neuronal cultures to select new chemical molecules pharmacologically active to prevent different types of neuronal pathologies,
- other studies concerning bovine, rabbit, human, articular chondrocytes cultures on the biomaterials comprising and/or consisting of hyaluronic acid derivatives.

The following tests had been carried out under my own responsibility.

## Experimental section

The object of this study was to determine the osteoinductivity and osteoconductivity of the multilayer composite material disclosed and claimed in the US Patent Application No. 10/580,659. Particularly, a matrix comprising HA, a HA derivative and DM (demineralised bone matrix) is sandwiched between two HA derivative layers, as claimed in the currently amended Claim1.

INTRODUCTION AND BACKGROUND:

Experimental samples were prepared in order to assess osteoinductivity and osteoconductivity in vivo. When implanted into normal animals, human DM is xenogeneic, and is expected to provoke an immune response that may compromise the analysis of osteoinduction. To avoid this, we used the athymic mouse model. The athymic mouse lacks a thymus gland and, therefore, cannot mount a humoral immune response to the human DM implants.

A total of 18 samples were implanted bilaterally into mouse hamstring muscle (see Table below). The hamstring muscle group (biceps femoris muscle) is a large, easily accessible muscle, which is commonly used as an implant site to evaluate heterotopic bone formation. Histological evaluation of the test articles was conducted at 28 days after implantation.

MATERIALS:

The multilayer composite material was made according to **Example 7 of the US Patent Application No. 10/580,659**, therefore, wherein:

- the inner matrix (I) was a paste obtained with Hyaff11p75 fibres (benzyl ester of HA having a percentage of esterification of 75%, see par. [0134]), hyaluronic acid and DM;
- said matrix (I) was sandwiched between two layers (II) of Hyaff11 (benzyl ester of HA, see par. [0134]) both in form of woven fabric;
- the so obtained multilayer material was calendered, freeze-dried, and then cut to size;
- the edges of the freeze-dried pieces were wetted with a solution of Hyaff11 in DMSO, and the process was continued to obtain the final dried multilayer composite material according to the current Claim 1.

Separately, a sponge of Hyaff11p75 has been prepared according to **Example 1 of Valentini et al.**, said sponge further entrapping DM within its pores, in the same amount and from the same lot as of the DM used for producing the claimed product above.

Additionally, heat inactivated DM putty has been provided as **negative control**, said DM being of same amount and from the same lot as of the DM used for producing the claimed product above and the product according to the prior art document.

A total of 18 samples were implanted and analyzed, wherein:

- Group **F1** included 8 samples of multilayer composite material according to the current Claim 1;
- Group **F2** included 5 samples of the sponge according to Valentini et al.; and
- Group **F3** included 5 samples of negative control material.

METHOD:

Osteoinductivity, that is the capability to produce heterotopic bone *de novo*, and osteoconductivity, that is the capability to favour cell migration within the scaffold, were assessed histologically, by following intramuscular implantation of the samples in an athymic mouse model.

Each sample weighed approximately 20 mg. The samples were randomized and implanted bilaterally in the hamstring muscles of athymic mice. Intramuscular implantation of multilayer composite material of the invention is expected to induce cartilage and then bone formation within the implants.

Animals were sacrificed at 28 days post-implantation.

Decalcified histology was then performed on the explanted samples; 5 histological slides with at least 2 sections per slide were prepared for each sample (at least 10 sections total per sample). Slides were stained with hematoxylin and eosin and a semi-quantitative scoring system was utilized to assess osteoinduction and osteoconduction using the scoring system described below.

The observer was blinded to the identification of the implant.

Osteoinductive and osteoconduction scores were based on the degree to which new bone, bone cells, osteoid, calcified cartilage remnants, and marrow elements are present.

The following scoring system was utilized:

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|---|---|
| 0 | No evidence of new bone formation |
| 1 | 1-25% of the section is covered by new bone |
| 2 | 26-50% of the section is covered by new bone |
| 3 | 51-75% of the section is covered by new bone |
| 4 | >75% of the section is covered by new bone |

The overall score for each implant was obtained by averaging the highest 5 scores from the histological slides; scores for each experimental group were determined by pooling the overall scores of the individual implants.

The results of semi-quantitative scoring are presented in the following Table.

Table. Osteoinduction and osteoconduction scores at 28 days post-implantation.

| Group | | Five best bone scores of Individual Histology Sections | | | | | | Group average |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | | |
| **F1** | F1-1 | 2 | 2 | 2 | 2 | 2 | 2.00 | |
| | F1-2 | 1 | 1 | 1 | 1 | 1 | 1.00 | |
| | F1-3 | 2 | 2 | 2 | 2 | 2 | 2.00 | |
| | F1-4 | 3 | 3 | 3 | 3 | 3 | 3.00 | |
| | F1-5 | 1 | 1 | 1 | 1 | 1 | 1.00 | **2.00** |
| | F1-6 | 2 | 2 | 2 | 2 | 2 | 2.00 | |
| | F1-7 | 3 | 3 | 3 | 3 | 3 | 3.00 | |
| | F1-8 | 2 | 2 | 2 | 2 | 2 | 2.00 | |
| F2 | F2-1 | 0 | 0 | 0 | 0 | 0 | 0.00 | |
| | F2-2 | 0 | 0 | 1 | 1 | 1 | 0.60 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | F2-3 | 0 | 0 | 0 | 0 | 0 | 0.00 | 0.24 |
| | F2-4 | 0 | 0 | 1 | 1 | 1 | 0.60 | |
| | F2-5 | 0 | 0 | 0 | 0 | 0 | 0.00 | |
| F3 | F3-1 | 0 | 0 | 0 | 0 | 0 | 0.00 | |
| | F3-2 | 0 | 0 | 0 | 0 | 0 | 0.00 | |
| | F3-3 | 0 | 0 | 0 | 0 | 0 | 0.00 | 0.00 |
| | F3-4 | 0 | 0 | 0 | 0 | 0 | 0.00 | |
| | F3-5 | N/A* | N/A* | N/A* | N/A* | N/A* | N/A* | |

* N/A indicates no score obtained due to histological artefacts on slides.

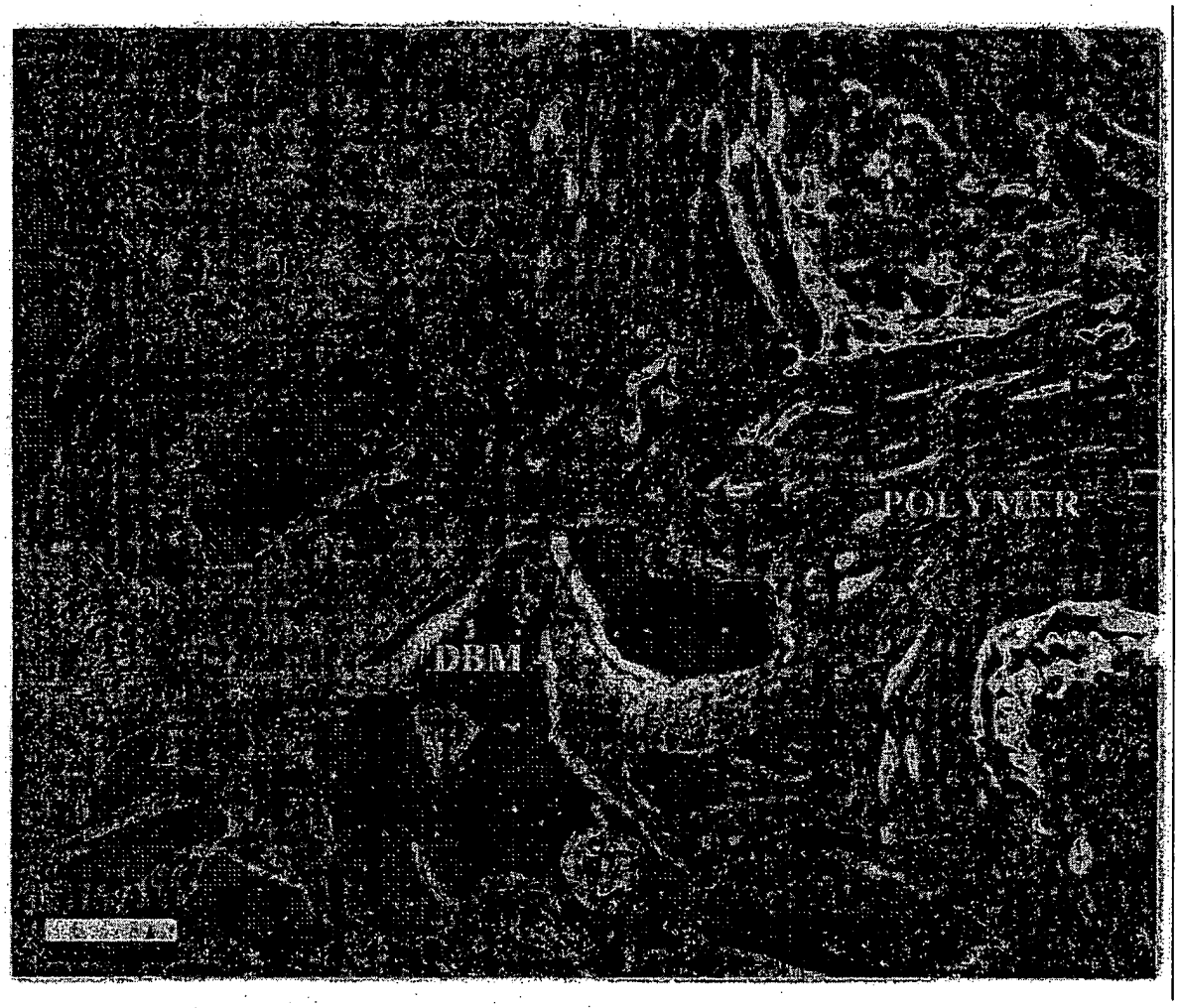
From the results in the above Table, it can be seen that the samples of multilayer composite material according to the invention (**F1**) were osteoinductive **and** osteoconductive, with an average bone score of 2.0. Conversely, the samples of sponge according to Valentini et al. (F2) had a definitely unsatisfactory average bone score of 0.24, considering that the negative control DM (F3) produced no bone, with bone scores of '0'.

Particularly, it should be noted that, for the sponges of Valentini et al., the HYAFF polymer was visible in the histology slides indicating that the polymer had not completely resorbed by 28 days post-implantation in the muscle pouch. It has been, indeed, observed an inflammation and no bone formation in the HYAFF polymer-rich regions of the sponges.

As far as the multilayer composite material according to the invention (**F1**) is concerned, the following Figure 1 and Figure 2 have been herewith enclosed in order to demonstrate the surprising and advantageous osteoinductivity and osteoconductivity so achieved.

Figure 1. Group F1. H&E stain; 100X. This high magnification view shows a DM-rich region in the sample with the highest bone score. Note formation of new bone ossicle with marrow (arrow) associated with residual DM. BAR = 100 microns.

Figure 2. Group F1. H&E stain; 40X. This intermadiate magnification view again shows discrete region of DM and HYAFF polymer. BAR = 250 microns.



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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: July 21, 2009

Anna Zanellato